



# Glutathione (GSH) induces embryogenic response in in vitro cultured explants of *Arabidopsis thaliana* via auxin-related mechanism

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## Abstract

To gain insight into the relationship between oxidative stress and somatic embryogenesis (SE) induction, we analysed the effects of a reduced glutathione (GSH) of antioxidant activity on the embryogenic potential of in vitro cultured explants of the model plant *Arabidopsis*. The results showed that the GSH treatment resulted in SE induction in the explants that had been cultured on an auxin-depleted medium. The GSH-induced SE was shown to be associated with auxin biosynthesis and the accumulation of both the *YUCCA* transcripts (*YUC10/11*) and the indolic compounds that are indicative for IAA. Inversely, 2,4-D treatment was found to increase the GSH level in the cultured explants and thus, complex interactions between the auxin and oxidative stress were assumed to control SE induction. The genetic regulators of auxin-induced SE, the *LEC1* and *LEC2* genes, were also up-regulated in the GSH-triggered SE. In contrast, the expression profiles of *BBM* and *MYB118* were distinctly different in the GSH- than in 2,4-D-induced SE, which suggests differences in the genetic regulation of these alternative embryogenic pathways. Collectively, the study provides evidence that the GSH-imposed changes in the oxidative stress level affect auxin production, which triggers embryogenic development in the cultured explants.

**Keywords** *Arabidopsis thaliana* · Auxin biosynthesis · Glutathione · Oxidative stress · Redox · Somatic embryogenesis

## Introduction

Somatic embryogenesis (SE) is a unique plant-specific developmental process in which the differentiated somatic cells of plants produce somatic embryos (Jiménez 2001). SE is widely used in green biotechnology to regenerate plants from in vitro cultured tissue for genetic transformation, plant micropropagation and artificial seed production. Besides its practical value, SE provides an experimental system in studies on the genetic and physiological determinants of the developmental plasticity of plant somatic cells. In particular, investigations of SE induction in *Arabidopsis*, a model in plant genomics, have substantially contributed to identifying

the genes that control the embryogenic transition in somatic plant cells (Nowak and Gaj 2016).

In order to induce SE, explants are commonly treated with auxins and 2,4-D, an auxinic herbicide that is widely used to efficiently induce SE in different plants including *Arabidopsis* (Gaj 2004). In addition to its auxin-like activity, 2,4-D has also been postulated to trigger SE through a stress response-related mechanism (Fehér 2015). In line with this assumption, a differential expression of the stress-related genes in 2,4-D-induced embryogenic cultures of *Arabidopsis* (Gliwicka et al. 2013) and other plants (Thibaud-Nissen et al. 2003; Sun et al. 2012) was reported. Although numerous studies have demonstrated the role of stress responses in the embryogenic transition of somatic plant cells, the relationship between stress and the SE induction mechanism remains unclear (Zavattieri et al. 2010).

The oxidative stress that results from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defence seems to be inseparably associated with plant in vitro cultures (Fehér et al. 2003). A major role in the intracellular defence against oxidative stress was attributed to glutathione (GSH), a ubiquitous tripeptide of non-enzymatic antioxidant activity. GSH reduces the stress level by quenching

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ROS in the ascorbate–glutathione pathway and the ratio of the reduced (GSH) to oxidised (GSSG) glutathione indicates the cellular redox status (Foyer and Noctor 2011).

GSH acts as a multifunctional signalling molecule in various processes of plants including the detoxification of heavy metals (Freeman et al. 2004), the modulation of the cell cycle and cell differentiation (Vernoux et al. 2000), the regulation of cell apoptosis (Hall 1999), embryo development and seed maturation (Lim et al. 2011). GSH controls the activity of the ROS-specific proteins including those that are critical for gene transcription, and relevantly, GSH has been indicated to regulate gene expression in different organisms including *Arabidopsis* (Queval and Foyer 2012; Hacham et al. 2014).

The effect of GSH in in vitro plant cultures has been investigated and, most frequently, GSH has been used to regenerate plants that have an increased tolerance to different abiotic stresses (Qiu et al. 2013; Nahar et al. 2015). Rarely has the impact of GSH on plant morphogenesis that is induced in vitro been evaluated. GSH treatment was reported to reduce somatic embryo production in *Daucus carota* (Ernshaw and Jahson 1985), while it inhibited SE and improved shoot organogenesis in a culture of *Gladiolus hybridus* (Gupta and Datta 2003). In conifers, a low concentration of GSH promoted somatic embryo production and alterations in the glutathione redox state improved the quality of white spruce somatic embryos (Belmonte and Yeung 2004; Belmonte et al. 2005). It was postulated that in *Picea glauca*, GSH favored SE induction, while a more oxidised environment provided by GSSH treatment improved the differentiation and development of somatic embryos (Stasolla 2010). Similarly, in an embryogenic culture of *Araucaria angustifolia*, the effect of GSH differed between the early and advanced stages of SE (Vieira Ldo et al. 2012).

In *Arabidopsis*, studies on the role of the redox status in the embryogenic response of cultured explants are limited and include the genetic manipulation of ascorbic acid (AA) at a cellular level to improve the number and quality of somatic embryos (Becker et al. 2014). Thus, to learn more about the impact of oxidative stress on the embryogenic reprogramming of somatic cells and, in particular, to identify the genetic components that respond to the redox status of a culture, we analysed the in vitro morphogenic responses of GSH-treated explants of *Arabidopsis*, which is a model in plant genomics (Wójcikowska and Gaj 2016).

## Materials and methods

### Plant material

The Columbia (Col-0) genotype of *Arabidopsis thaliana* (L.) Heynh and transgenic plants with the reporter construct

in the Col-0 background were used. The Col-0 seeds were supplied by NASC (The Nottingham Arabidopsis Stock Centre,). The seeds of the *YUC* (*pYUC10-GFP*, *pYUC11-GFP*) were kindly provided by H el ene S. Robert (CEITEC Central European Institute of Technology, Masaryk University, Brno, Czech Republic). The seeds of *pLEC1-GFP* and *pBBM-GFP* reporter lines were kindly provided by Kim Boutilier (Wageningen University & Research, Wageningen, Netherlands) and the seeds of the *pLEC2-GFP* line were kindly provided by Anna W ojcik (Department of Genetics, University of Silesia, Katowice, Poland).

## In vitro cultures

### Explants and culture media

Immature zygotic embryos (IZEs) at the late cotyledonary stage of development were used to establish an in vitro culture following the standard protocol for explant isolation and sterilisation (Gaj 2001). All of the media that were used for the explant culture were based on an E0 medium that consisted of B5 micro- and macro-elements (Gamborg et al. 1968), 20 g/L sucrose and 8 g/L agar. Different modifications of the E0 medium were applied that included the supplementation of E0 with (i) 0.1, 0.5 and 0.75 mM of GSH (Glutathione L-reduced, Sigma-Aldrich) (E0G); (ii) 5  $\mu$ M of 2,4-D (2,4-dichlorophenoxyacetic acid; Sigma-Aldrich) (E5); (iii) 5  $\mu$ M of 2,4-D and GSH at concentration of 0.1 and 0.5 mM (E5G). The GSH was dissolved in an H<sub>2</sub>O, filter sterilised and added to the autoclaved and cooled down E0 and E5 media to produce an E0G and E5G medium, respectively. The freshly prepared GSH-media were used for the explant culture on the same day.

### Evaluation of the morphogenic capacity of the cultures

The morphogenic responses of the explants that had been cultured for 3 weeks were evaluated. In the E0 culture, the percentage of explants that developed seedlings was scored. In E5 culture, two parameters were estimated: SE efficiency (the percentage of explants that formed somatic embryos) and SE productivity (the average number of somatic embryos produced per explant). Ten explants were cultured in one Petri dish ( $\varnothing$  60 mm) and thirty explants were analysed in one replicate. A total of three replicates (90 explants) were analysed per one culture combination.

### Plant and in vitro culture growth conditions

The plants were grown in soil pots in a “walk-in” type green room under controlled conditions (20–22 °C, 16/8 h L/D photoperiod, light intensity of 100  $\mu$ E/m<sup>2</sup>s). The explants were cultured in a growth chamber under

controlled conditions (20–22 °C, 16/8 h L/D photoperiod, light intensity of 40  $\mu\text{E}/\text{m}^2\text{s}$ ).

### Total RNA isolation

An RNAqueous kit (Ambion) was used to isolate the total RNA from the different culture combinations, which included the explants that had been induced on the E0, E5 and E0 media with 0.5 mM GSH (E0G). The explants that had been induced on the E0G medium that were capable of SE induction (E0G-E) were analysed separately from those that were incapable of the embryogenic transition (E0G-N). The tissue were sampled on days 0, 10 15 of the culture. The RNA concentration and purity were measured using a Nano-Drop ND-1000 (NanoDrop Technologies).

### Reverse transcription and real-time qPCR reaction

To control any DNA contamination, RNA was treated with RQ1 RNase-free DNase I (Promega) according to the manufacturer's instructions. First strand cDNA was produced in a 20  $\mu\text{L}$  reaction volume using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The product of the reverse transcription was diluted with water at a 1:4 ratio and 2.5  $\mu\text{L}$  of this solution was used for the RT-qPCR reactions. RT-qPCR was carried out in a 10  $\mu\text{L}$  reaction volume using a LightCycler<sup>®</sup>480 SYBR Green I Master (Roche). The expression pattern of the genes were established using LightCycler<sup>®</sup> 480 System (Roche) and a commercial LightCycler<sup>®</sup> 480 SYBRTM Green I Master kit (Roche), LightCycler<sup>®</sup> 480 Multiwell Plate 96 and Multiwell Sealing Foil (Roche). The relative RNA levels were calculated and normalised to the internal controls, *AT4G27090* gene encoded 60S ribosomal protein (Thellin et al. 1999). RT-qPCR analyses were carried out in three biological repetitions with two technical replicates of each repetition. Fold change values were calculated using the comparative  $2^{-\Delta\Delta\text{Ct}}$  method where  $\Delta\Delta\text{Ct}$  represents  $\Delta\text{Ct}^{\text{reference condition}} - \Delta\text{Ct}^{\text{compared condition}}$ , and  $2^{-\Delta\Delta\text{Ct}}$  indicates normalized expression ratio (Livak et al. 2013).

### Microscope analysis

The GFP signal was analysed with the use of a Nikon Eclipse Ni-E/Ni-U fluorescent microscope system. GFP fluorescence was excited using a metal halide lamp (Prior Lumen200) with a wavelength of 488 nm. Photographic documentation was created from images that were recorded with a Nikon Digital Sight DS-Fi2 camera with DS-U3 Controller.

### Analysis of the indolic compound content

A colourimetric technique that enables the detection of indolic compounds including IAA was used (Bric et al. 1991). Fresh tissues (25–80 mg) were collected from the cultures that had been induced on the E0, E5 and E0G media for 10 and 15 days. The explants that were capable of SE induction on the E0G medium (E0G-E) and those that were incapable of SE on this medium (E0G-N) were analysed separately. The tissue samples were transferred to mortars containing 2 mL of 10 $\times$ PBS and kept at 4 °C. The material was homogenised and the solution was centrifuged (25 min; 18,000 $\times g$ ). Two mL of supernatant was mixed with 100  $\mu\text{L}$  of 10 mM orthophosphoric acid and 4 mL of Salkowski' reagent (150 mL H<sub>2</sub>SO<sub>4</sub>; 250 mL ddH<sub>2</sub>O; 7.5 mL 0.5 M FeCl<sub>3</sub>). The absorbance was read at 530 nm after a 30-min incubation at room temperature. The concentrations of the indolic compounds, which is indicative of the IAA content, was determined using the calibration curve of pure IAA as the standard following the linear regression analysis. Each analysis was carried out in three biological replicates and two technical replicates.

### Evaluation of the GSH content

To analyse the GSH content, 15 mg of the tissues were collected from the explants that had been induced on the E0, E5 and E0G media for 10 and 15 days. The GSH concentration was estimated using the spectrophotometry method (Anderson 1985) in which the calibration curve of pure GSH as the standard was used following the linear regression analysis. Each analysis was carried out in three biological replicates and two technical replicates.

### Statistical analysis

To calculate the significant differences (at  $P < 0.05$ ) between the compared samples, the Tukey's HSD test was used. The figures show the averages from the biological replicates with their standard deviation.

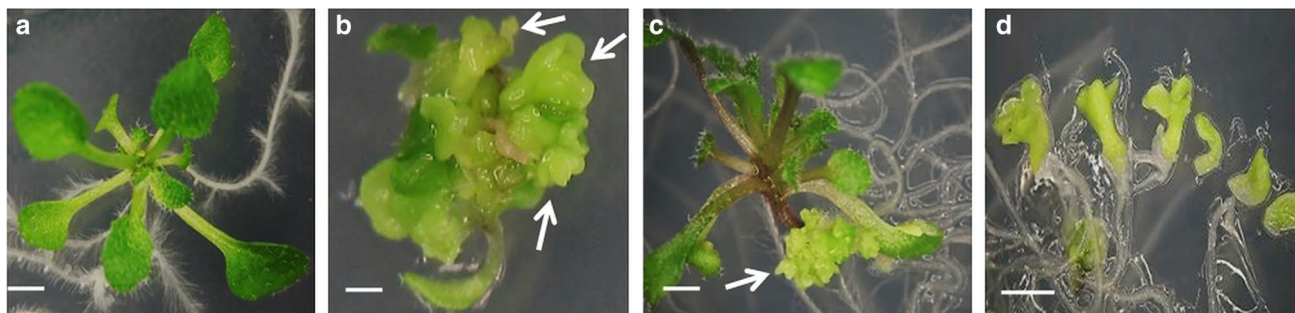
## Results

### Explant treatment with glutathione (GSH) results in SE induction

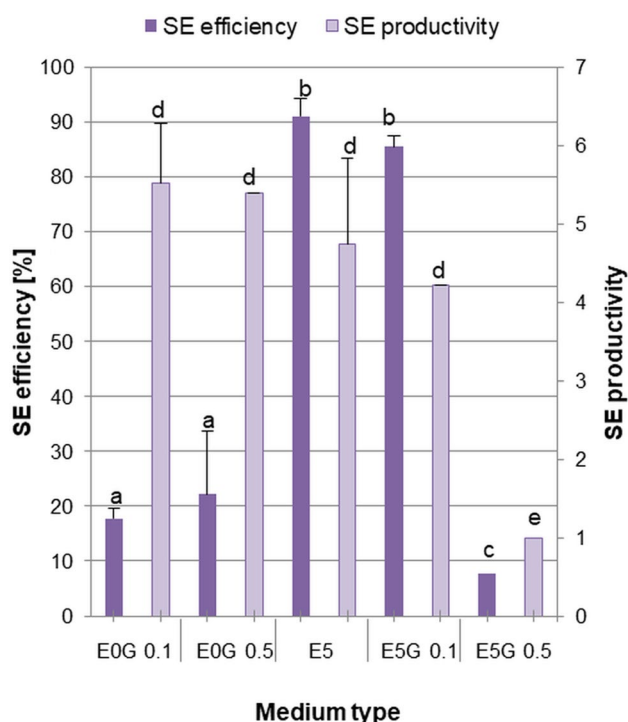
In order to assess the role of oxidative stress in the embryogenic response of plant tissues that are cultured in vitro, GSH was used to treat the Arabidopsis explants of immature zygotic embryos. The explants were cultured on an E0 medium that was supplemented with different GSH concentrations (0.1; 0.5 and 0.75 mM). We found that the

treatment with 0.75 mM of GSH was highly toxic to the explants and most of them (over 95%) died. In contrast, the explants that had been treated with 0.1 and 0.5 mM of GSH developed into seedlings the majority of which displayed a normal phenotype (Fig. 1a), while about one-fifth of the seedlings showed severe developmental malformations. A closer inspection of these seedlings revealed the inhibition of the shoot and root development and the formation of a callus and embryo-like structures on different seedling organs including the cotyledons, hypocotyl and SAM proximity (Fig. 1b, c). The GSH-induced embryo-like structures that had been isolated from the explant tissue were capable of normal root development, which confirmed the bipolar embryo identity of these structures (Fig. 1d). To evaluate embryogenic effectiveness of the GSH, explants that had been treated with 0.1 (E0G0.1) and 0.5 (E0G0.5) mM of GSH were analysed in terms of the percentage of embryogenic explants (SE efficiency) and the average number of somatic embryos that had been produced per embryogenic explant (SE productivity) (Fig. 2). The results showed that both media affected the explant response on E0 medium in a similar manner. Accordingly, 18 and 22% of the explants that had been treated with 0.1 and 0.5 mM of GSH, respectively, were found to be embryogenic and they produced 5.4 somatic embryos per explant on average.

In addition, the effect of GSH on the explants that had been induced by 2,4-D was examined. To this end, an E5 medium with 5  $\mu$ M of 2,4-D, which is routinely used to induce SE in Arabidopsis, was supplemented with 0.1 and 0.5 mM of GSH. The results indicated that the presence of GSH distinctly inhibited the effective SE response that was induced on the E5 medium. In particular, GSH at a concentration of 0.5 mM drastically reduced the number of embryogenic explants (from 92% on E5 to 8%) and most (92%) of the E5G0.5-induced explants produced non-embryogenic calli.



**Fig. 1** GSH treatment results in the SE induction. The IZE explants that had been cultured for 21 days on the E0 medium that had been supplemented with 0.5 mM of GSH medium developed into normal (a) and malformed (b, c) seedlings with embryo-like structures. The



**Fig. 2** Embryogenic potential of the IZE explants treated with GSH. The IZE explants were cultured for 21 days on an auxin-free E0 medium that had been supplemented with GSH at concentrations of 0.1 (E0G 0.1) and 0.5 (E0G 0.5) mM; 2,4-D at concentration of 5  $\mu$ M (E5) and an E5 medium with 0.1 (E5G 0.1) and 0.5 (E5G 0.5) mM of GSH. SE efficiency (the percentage of the explants that developed somatic embryos) and SE productivity (the average number of somatic embryos per embryogenic explant) was evaluated. Different letters indicate significantly different values (Tukey's HSD test,  $P < 0.05$ ). Error bars indicate standard deviation (SD)

Taken together, the results showed that GSH is potent enough to induce an SE response on an auxin-free medium while it negatively affects the embryogenic response that is induced by 2,4-D.



## Culture combinations subjected to an analysis of the GSH-induced SE

Given that treating the explants with 0.5 mM of GSH resulted in an embryogenic response on the auxin-free E0 medium and the strongly impaired embryogenic response induced by 2,4-D, the explants that had been cultured on E0 + 0.5 mM GSH (E0G) were subjected to an analysis of the molecular mechanism that is involved in GSH-induced SE. Considering that only about one-fifth of the GSH-treated explants was capable of an embryogenic response, the E0G cultures on the 10th day were closely examined and embryogenic (E0G-E) and non-embryogenic (E0G-N) explants were selected for the analysis (Fig. S1, supplementary data). In addition to the GSH-induced explants, the analysis also included the explants that had been cultured on the 2,4-D-free E0 medium (non-embryogenic negative control) and the E5 medium with 2,4-D (highly embryogenic positive control), which had resulted in seedling development and efficient SE induction, respectively.

## GSH treatment activates the auxin-biosynthesis pathway

### GSH treatment up-regulates the *YUC10* and *YUC11* genes

To investigate whether auxin biosynthesis might be involved in the GSH-promoted embryogenic transition, we profiled the expression of eleven *YUCCA* genes encoding key enzymes of the auxin biosynthesis pathway in Arabidopsis.

The results of the RT-qPCR analysis indicated that two of the analysed genes, including *YUC10* and *YUC11*, were significantly up-regulated in the GSH-induced explants (Fig. 3a). The *YUC10* transcripts were highly accumulated (up to 45-fold) on day 15 of the culture, while *YUC11* expression level was distinctly increased (up to sevenfold) in the early culture (10 day). In contrast to *YUC10* and *YUC11*, the other *YUC* genes were either not expressed (*YUC2* and *YUC5*) or their transcripts were down-regulated (*YUC1*, *YUC2*, *YUC3*, *YUC4*, *YUC5*, *YUC6*, *YUC7*, *YUC8*, *YUC9*) in response to GSH (Fig. S2, Supplementary Data).

### GFP-monitored *YUC10* and *YUC11* expression

The involvement of the *YUC10* and *YUC11* genes in the GSH-induced embryogenic response was further investigated using the *YUC10::GFP* and *YUC11::GFP* reporter lines (Fig. 3b). In the freshly isolated explants, the GFP signal for *YUC10* and *YUC11* was not detected. On day one of the treatment with GSH, a strong expression of *YUC10* was indicated in the hypocotyls and cotyledons of 24% of the explants (Tab. S1 supplementary data). During the culture, the *YUC10* expression pattern was distinctly changed and on

the 5th and 10th day the GFP-*YUC10* signal was limited to small patches that were dispersed on the adaxial side of the cotyledons of 22–23% of the explants. In contrast to *YUC10*, the GFP-*YUC11* signal was detected only in the late culture and on the 10th day of the GSH treatment, about 23% of the explants displayed a *YUC11* expression in isolated areas located on the cotyledons and in the proximity of the SAM.

## An accumulation of auxin and GSH is associated with SE induction

In line with the finding that GSH-treatment resulted in the up-regulation of the auxin-biosynthesis *YUC10* and *YUC11* genes, we also observed a substantial increase in the indolic compound (IC) content in the GSH-induced embryogenic culture (Fig. 4a). Accordingly, the IC content was 2–3 times higher in the embryogenic (E0G-E) culture than in the non-embryogenic cultures, including the explants that were incapable of SE induction on the GSH-medium (E0G-N) and the explants that had developed into seedlings on the PGR-free (E0) medium. In conclusion, analysis of the IC content supported an assumption that similar to the SE-induction on the medium with 2,4-D (E5), the GSH-induced embryogenic response was associated with the auxin accumulation in the explants.

To further explore the relationship between GSH and auxin during the embryogenic response, we evaluated the level of GSH in the explants that were cultured on the E0, E0G and E5 media (Fig. 4b). The analyses indicated a significant increase in the GSH content in the SE-induced explants in response to the treatments with both GSH and 2,4-D. The positive effect of the 2,4-D treatment on the GSH content in the explants infers a role of GSH in an auxin-related mechanism of SE induction.

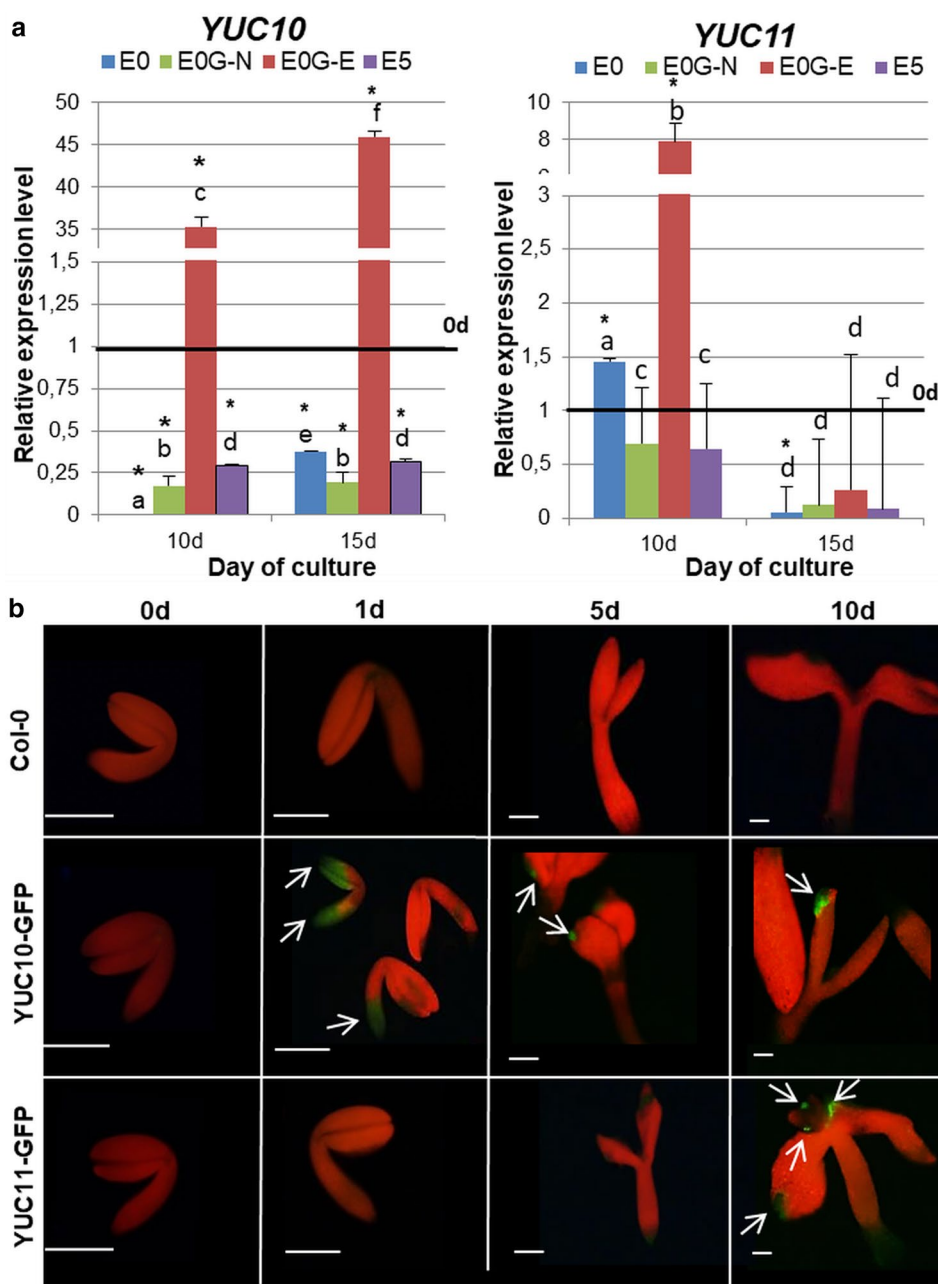
## Expression of the SE-involved TF genes in the GSH-treated explants

In order to gain insight into the genetic regulation of GSH-induced SE and its similarity to the auxin-triggered SE pathway, we analysed the expression of *LEC1*, *LEC2*, *BBM* and *MYB18*, which encode the TFs that are engaged in auxin-induced SE in Arabidopsis (Nowak and Gaj 2016). Accordingly, the level and pattern of gene expression was analysed using RT-qPCR and reporter lines.

### RT-qPCR analysis

The expression level of the *LEC1*, *LEC2*, *BBM* and *MYB18* genes was monitored in the explants that had been cultured for 10 and 15 days on the E0G, E0 and E5 media. The results showed that the GSH treatment resulted in a significantly increased expression of the *LEC1*, *LEC2* and

**Fig. 3** Expression level and pattern of the *YUC10* and *YUC11* genes in the GSH-treated explants. The expression level of the *YUC10* and *YUC11* genes was analysed using RT-qPCR (a). The explants had been cultured on the E0, E0G (E0+0.5 mM GSH) and E5 (E0+5  $\mu$ M 2,4-D) media and the embryogenic (E0G-E) versus non-embryogenic (E0G-N) explants that had been induced on the E0G medium were analysed. The relative transcript level was normalised to the internal control (*At4g27090*) and calibrated to the 0 day of culture. Asterisk indicates values that are significantly different from 0 day culture; different letters indicate values that differ significantly between compared culture combinations (Tukey's HSD test,  $P < 0.05$ ). Error bars indicate the standard deviation (SD). The GFP-monitored spatio-temporal expression of the *YUC10* and *YUC11* genes (b). Col-0 was used as a negative control. GFP signal was analysed in 0, 1, 5 and 10 days cultures. Arrows show GFP signal; bars = 1 mm

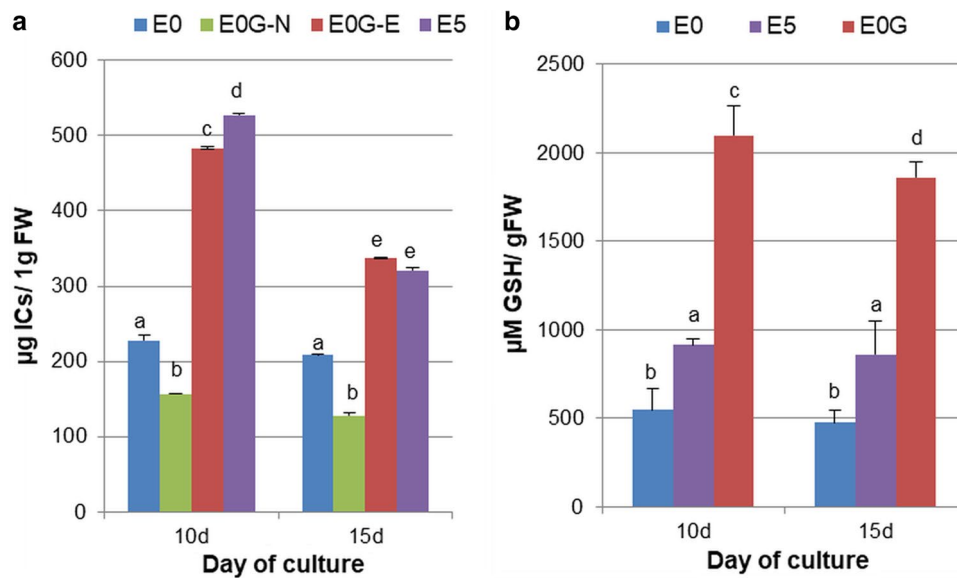


*MYB118* transcripts (Fig. 5a). In particular, *MYB118* was intensively up-regulated (up to 70–239-fold) in the E0G-E explants that exhibited over three-fold higher level of the *TF* gene transcripts than the non-embryogenic culture (E0G-N). Notably, the high expression of the *MYB118* gene seems to be specific for the GSH-induced SE as the embryogenic culture induced by 2,4-D (E5) had a low accumulation of the gene transcripts. In general, the non-embryogenic cultures, including E0G-N and E0, demonstrated a substantially lower transcript level of the *LEC1*, *LEC2* and *MYB118* genes compared to the E0G-E culture. In contrast, a significant increase in the *BBM* expression was observed exclusively in the explants that had been cultured on the E5 medium.

A low level of the *BBM* transcripts, similar to that observed in E0 culture, was characteristic of the GSH-treated of both the embryogenic and non-embryogenic explants (E0G-E and E0G-N).

#### Analysis of the GFP-reporter lines

In addition to the RT-qPCR analysis, the GFP reporter lines were used to localize the expression of the *LEC1*, *LEC2* and *BBM* transcripts in the explants in response to GSH. In the freshly isolated explants, no *TF*-indicative GFP signal was detected (Fig. 5b). In contrast, the expression of the *TF* genes was rapidly induced by GSH and 23–24% of the



**Fig. 4** Concentration of indolic compounds (ICs) and GSH in the GSH- and 2,4-D-treated explants, respectively. Increased content of ICs during GSH-induced SE (a). IZE explants of Col-0 had been cultured on the E0+0.5 mM GSH (E0G), E0 and E0+5 µM 2,4-D (E5) media. Two explant types were analysed in the culture on the E0G medium: the embryogenic (E0G-E) and non-embryogenic (E0G-N) explants. Different letters indicate values that are significantly dif-

ferent (Tukey's HSD test,  $P < 0.05$ ). Error bars indicate the standard deviation (SD). Increased content of GSH during 2,4-D-induced SE (b). IZE explants of Col-0 that had been cultured on the E5 (E0+5 µM 2,4-D), E0 and E0G (E0+0.5 mM GSH) media. Values that were significantly different from E0 (a); E5 (b) were indicated (Tukey's HSD test,  $P < 0.05$ ). Error bars indicate the standard deviation (SD)

explants showed a GFP signal (Tab. S1, supplementary data) at day 1 of the culture on the E0G medium. The localisation of the GFP signal was remarkably changed during the next days of the culture and it became restricted to the SE-involved explant parts. Accordingly, the explants that had been cultured on the GSH-medium for 5 days had a GFP signal at isolated locations that were dispersed on the adaxial side of the cotyledons (*LEC1* and *LEC2*) and in proximity of the SAM (*BBM*). In support of the SE-related expression of the analysed *TFs*, a similar fraction of the explants displayed a GFP signal (22–24%; Tab. S1, supplementary data) and were capable of SE induction on the GSH-medium (22%, Fig. 2). Hence, the early induction of the *TF* genes expression seems to be associated with a GSH-triggered embryogenic response.

In conclusion, the results indicate that GSH-treatment induces an SE response in the somatic tissue of Arabidopsis via an auxin-related mechanism. In support of this hypothesis, the up-regulation of the *YUC* (*YUC10* and *YUC11*) genes that are involved in the auxin biosynthesis pathway and the accumulation of indolic compounds (indicative for IAA) were found in the GSH-treated explants. In addition, the up-regulation of the *LEC1* and *LEC2* genes in the GSH-treated embryogenic explants implies a similarity of the GSH-induced SE pathway to the one that was triggered by auxin (2,4-D). However, the contrasting expression profiles of *BBM* and *MYB118* that were found in the GSH- and

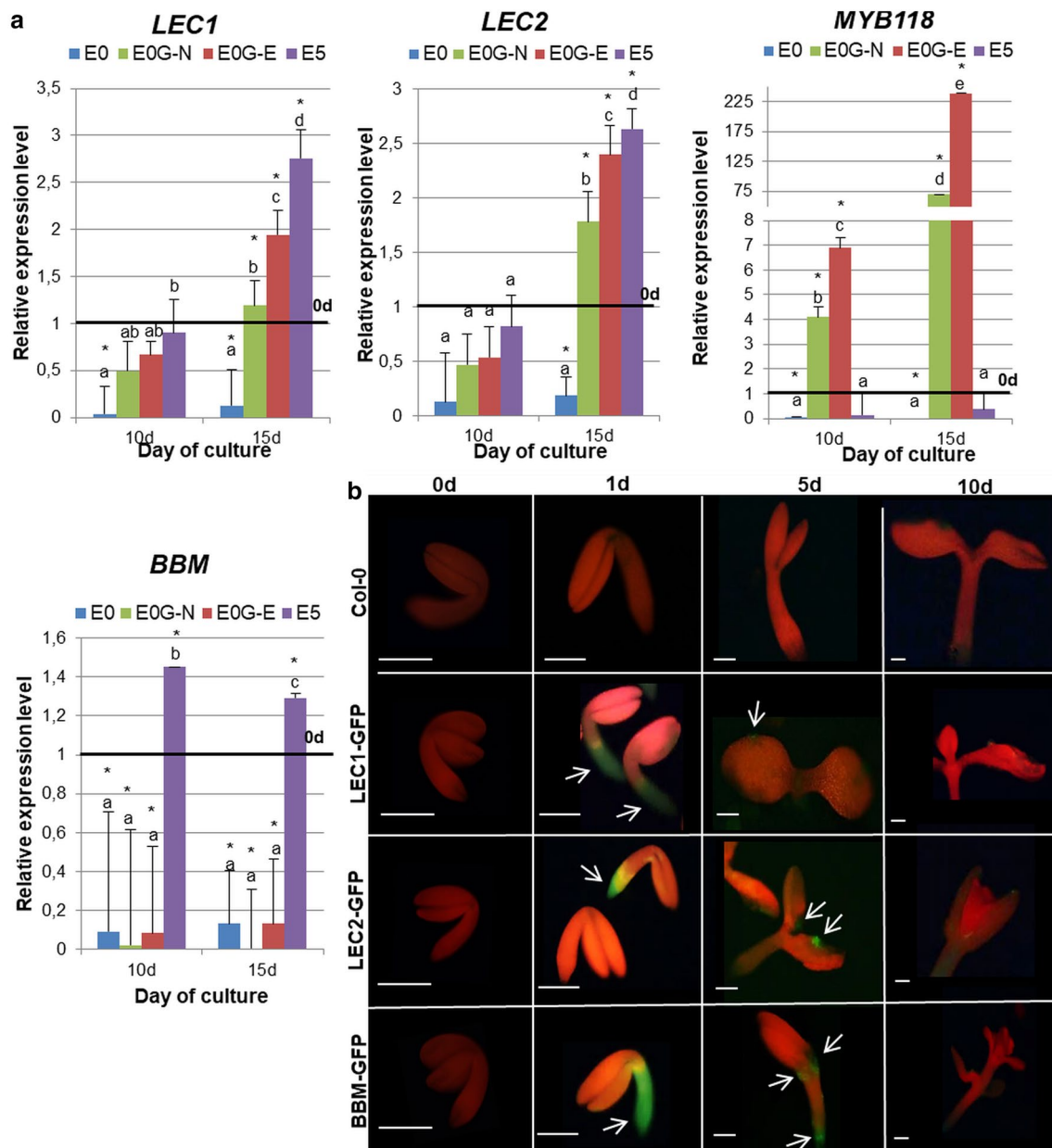
2,4-D-induced embryogenic cultures infer differences in the genetic regulation of SE that is triggered by these treatments. Moreover, the increased GSH content that was found in the embryogenic culture that was induced by 2,4-D further confirms the role of GSH in the embryogenic response of somatic plant cells.

## Discussion

### GSH is potent in inducing SE

In Arabidopsis, efficient SE induction requires the treatment of IZE explants with auxin, preferentially with 2,4-D (Gaj 2004). Here, we indicated that GSH is sufficient to induce SE in explants that have been cultured on an auxin-free medium. Although the beneficial effect of GSH on somatic embryo production in conifers was demonstrated (Belmonte and Yeung 2004), the SE-promoting action of GSH on an auxin-depleted medium has not yet been demonstrated. Although similar to 2,4-D, GSH promotes SE induction, the embryogenic processes that are triggered by these compounds seem to differ in terms of the type of SE-involved tissues and the efficiency of the responding explants.

In the 2,4-D-treated IZEs explants, the seedling development was inhibited and SE was efficiently induced by 80–90% of the explants and thus this system is extensively



**Fig. 5** Expression level and pattern of the *TF* genes in the GSH-treated explants. The expression level of the *LEC1*, *LEC2*, *MYB118* and *BBM* genes was analysed using RT-qPCR (a). The explants had been cultured on the E0, E0G (E0+0.5 mM GSH) and E5 (E0+5  $\mu$ M 2,4-D) media and the embryogenic (E0G-E) versus non-embryogenic (E0G-N) explants that had been induced on the E0G medium were analysed. The relative transcript level was normalised to the internal control (*At4g27090*) and calibrated to the 0d of

the culture. Asterisk indicates values that are significantly different from 0 day culture; different letters indicate values that differ significantly between compared culture combinations (Tukey's HSD test,  $P < 0.05$ ). Error bars indicate the standard deviation (SD). GFP-monitored spatio-temporal expression of the *LEC1*, *LEC2* and *BBM* genes (b). Col-0 was used as a negative control. GFP signal was analysed in 0, 1, 5 and 10 day cultures. Arrows show GFP signal; bars = 1 mm

used in studies on SE (Pillon et al. 1996; Gaj 2001; Ikeda-Iwai et al. 2002; Su et al. 2009; reviewed in Wójcikowska and Gaj 2016). The 2,4-D-induced somatic embryos were induced on the embryonic tissues of the IZEs including the adaxial side of the cotyledons and the proximity of

the SAM (Kurczyńska et al. 2007). In contrast to 2,4-D, GSH appeared to be potent in promoting the embryogenic response in the post-embryonic tissues of the IZE-derived and morphologically defective seedlings that had developed from the GSH-treated explants.



## A GSH-promoted SE is associated with auxin biosynthesis

We found that the supplementation of a medium with 2,4-D inhibited the SE-inductive effect of GSH and resulted in the production of a non-embryogenic callus in the IZE explants. We assumed that the explants that had been simultaneously treated with 2,4-D and GSH might over-accumulate auxin as explants treated with high 2,4-D concentrations showed a similar response (Wójcikowska et al. 2013). In line with the assumption that auxin was produced in the GSH-treated explants up-regulation of the *YUC* genes (*YUC10* and *YUC11*) that encode flavin monooxygenases of key function in the tryptophan-dependent pathway of IAA biosynthesis (Cheng et al. 2006) was indicated in the GSH-induced culture. *YUC10* and *YUC11* contribute to auxin synthesis during zygotic embryogenesis (Cheng et al. 2007) and the *YUC10* expression has also been associated with SE that is induced in the 2,4-D- and TSA-treated IZEs (Wójcikowska et al. 2013, 2018).

The GSH-induced auxin biosynthesis that was observed in the present study provides further evidence on the intensive crosstalk between oxidative stress and auxin, which control a cell's responsiveness to different developmental stimuli (Tognetti et al. 2012). GSH was suggested to control auxin homeostasis by impacting the signal transduction and transport of auxin in the roots of *Arabidopsis* (Vernoux et al. 2000; Koprivova et al. 2010). Although the interdependency between auxin and the stress responses in the control of plant development has been well documented, the understanding of how the auxin metabolism and signalling is regulated by stress and redox shifts remains rather limited (Tognetti et al. 2017). The ROS that are under the control of GSH and other components of the antioxidant machinery seem to provide important regulatory signals for the auxin-related processes, including auxin biosynthesis (Tognetti et al. 2012, 2017). Thus, the ROS that are accumulated during SE induction in *Arabidopsis* (Wójcikowska et al. 2018) and other plants (Luo et al. 2001; Ma et al. 2012) might affect the SE-associated auxin biosynthesis by various means, including the oxidation of the auxin precursors and lipid peroxidation (Bartel et al. 2001) and the regulation of the stress-regulated *YUC* genes (Liu et al. 2016). Identifying the stress-related TFs that bind to the promoters of the *YUC* genes will help to decipher the genetic components of the stress-regulated auxin biosynthesis that is involved in SE induction. In support of the stress-controlled expression of *YUC* genes, a binding site motif for stress-responsive MYB TF (MYB4) was identified in the promoter site of the *YUC10* gene (Ambawat et al. 2013; <http://arabidopsis.med.ohio-state.edu/AtcisDB/>).

We found that in parallel to a GSH-promoted auxin accumulation, the GSH level was substantially elevated in the 2,4-D-treated explants. Similarly, auxin treatment

was reported to elevate the GSH content in the cultures of *Daucus carota* (Ernshaw and Jahnson 1987) and *Medicago sativa* (Pasternak et al. 2014) and this effect might be attributed to the increased ROS production that accompanies the 2,4-D treatment of plant tissues both, in vivo (Rodríguez-Serrano et al. 2014) and in vitro (Elhiti and Stasolla 2015; Wójcikowska et al. 2018). The accumulating ROS promote the activity of glutamate-cysteine ligase (GCL), which is a rate-limiting enzyme in glutathione production, to control the redox balance (Foyer and Noctor 2011). In conclusion, as in other the developmental processes of plants, the feedback regulatory loop between 2,4-D/auxins and ROS/GSH is assumed to balance the level of oxidative stress during SE induction.

## The genetic regulators of the GSH-induced SE

To gain insight into the regulatory pathway of the GSH-triggered embryogenic response, the expression of the genes encoding the TFs that have a documented function in SE induction, including *LEC1*, *LEC2*, *BBM* and *MYB118* (Lotan et al. 1998; Stone et al. 2001; Boutilier et al. 2002; Gaj et al. 2005; Wang et al. 2009) were profiled. The results showed that GSH substantially up-regulated three of the analysed TFs, including *LEC1*, *LEC2* and *MYB118*. *LEC1* and *LEC2*, which are members of the *LEAFY COTYLEDON* group of genes that encode the master regulators of the hormone-related pathways during zygotic embryo development. An ectopic expression of *LEC1* and *LEC2* triggered SE induction in seedlings without auxin treatment (Lotan et al. 1998; Stone et al. 2001) and the essential role of these genes in the auxin-induced SE was demonstrated (Gaj et al. 2005). The present analysis suggests that *LEC1* and *LEC2* also contribute to GSH-induced SE possibly through the activation of a *YUC*-dependent auxin biosynthesis pathway (Stone et al. 2008). In support of this assumption, *YUC10* with a GSH-induced expression (present study) was postulated to be among the *LEC1* and *LEC2* targets (Wójcikowska et al. 2013).

Recently, *BBM* (*BABY BOOM*) was indicated to transcriptionally activate *LEC1* and *LEC2* during auxin-induced SE (Horstman et al. 2017). *BBM* encodes the TF of the AP2/ERF gene family and its overexpression promotes somatic embryo formation possibly through the promotion of cell division and growth (Boutilier et al. 2002; Passarinho et al. 2008). However, we found the *BBM* gene to be down-regulated in the GSH-induced embryogenic tissues and similarly, a low activity of this gene was also displayed in a TSA-induced embryogenic culture (Wójcikowska et al. 2018). These findings suggest that the function of *BBM* in SE induction might be dependent on the induction factors of SE.

Additional evidence on the differences in the genetic pathways that govern GSH- and 2,4-D-triggered embryogenic development were provided by the expression profiling of *MYB118*. *MYB118* encodes the MYB transcription factor whose overexpression promotes embryonic reprogramming in the root cells of *Arabidopsis* (Wang et al. 2009). We found that in contrast to a low expression of *MYB118* in the 2,4-D-treated explants, the gene was significantly up-regulated under GSH-treatment and, that in particular, its high overexpression was observed in the explants undergoing SE induction. The high expression of *MYB118* during GSH-induced SE might be attributed to the stress-related functions of this gene that are connected with the promotion of the biosynthesis of the storage compounds in the seeds (Barthole et al. 2014). Relevantly, seed maturation products were postulated to provide a stress-protective environment that enhances the embryogenic response of explants (Stone et al. 2008; Gliwicka et al. 2012). Importantly for the possible targets of *MYB118* in SE, many MYB binding sites are present in the promoter sites of the *LEC1* and *LEC2* genes that have key functions during zygotic and somatic embryogenesis (<http://arabidopsis.med.ohio-state.edu/AtcidsDB/>; Stone et al. 2008).

Collectively, the gene expression profiling implies that the GSH-induced embryogenic pathway shares some similarity to the one induced by 2,4-D in terms of the stimulation of the auxin-biosynthesis *YUC* genes and enhanced expression of the TF regulators of SE, *LEC1* and *LEC2*. However, the opposing expression profiles of *BBM* and *MYB118* that were found in the GSH- versus 2,4-D-induced SE implies a difference between the genetic pathways that are triggered in response to oxidative stress and auxin. Insights into the relationship between the expression of the TF genes and the redox balance would be helpful in defining the specific components of the GSH-triggered SE pathway.

In conclusion, the complex and mutual interactions between the redox status and genetic/epigenetic processes seem to control the embryogenic reprogramming of somatic plant cells and extensive studies are required to reveal the versatile roles of ROS and antioxidants in the molecular mechanisms of SE induction.

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